Maternal effects on offspring size in a natural population of the viviparous tsetse fly

Sinead English[1*], Honor Cowen[1], Emma Garnett[1] and John Hargrove[2]

Affiliations

1. Behavioural Ecology Group, Department of Zoology, University of Cambridge, Cambridge, UK

2. Centre of Excellence in Epidemiological Modelling and Analysis (SACEMA), University of Stellenbosch, Stellenbosch, South Africa

*Corresponding author:

Address: Department of Zoology, University of Cambridge, Downing Street, Cambridge, United Kingdom CB2 3EJ

Phone: +44 1223 331759

Email: sineadenglish@cantab.net

Abstract

1. Theory predicts that mothers should adaptively adjust reproductive investment depending on current reserves and future reproductive opportunities. Females in better intrinsic state, or with more resources, should invest more in current reproduction than those with fewer resources. Across the lifespan, investment may increase as future reproductive opportunities decline, yet may also decline with reductions in intrinsic state.

2. Across many species, larger mothers produce larger offspring, but there is no theoretical consensus on why this is so. This pattern may be driven by variation in maternal state such as nutrition, yet few studies measure both size and nutritional state, or attempt to tease apart confounding effects of size and age.

3. Viviparous tsetse flies (*Glossina* species) offer an excellent system to explore patterns of reproductive investment: females produce large, single offspring sequentially over the course of their relatively long life. Thus, per-brood reproductive effort can be quantified by offspring size.

4. While most tsetse reproduction research has been conducted on laboratory colonies, we investigated maternal investment using a unique field method where mothers were collected as they deposited larvae, allowing simultaneous mother-offspring measurements under natural conditions.

5. We found that larger mothers and those with higher fat content produced larger offspring, and there was a trend for older mothers to produce slightly larger offspring.

6. Our results highlight the importance of measuring maternal nutritional state, rather than size alone, when considering maternal investment in offspring. We also discuss implications for understanding vector population dynamics.

Keywords: maternal investment, state dependent, life history, ageing, terminal investment, viviparity, Diptera

Introduction

Life-history theory predicts that mothers should adjust their investment in reproduction according to their current state and expected future reproductive output (Williams, 1966; Pianka & Parker, 1975; McNamara & Houston, 1996). As individuals reach the end of their life, they might increase their investment in reproduction disproportionately as their chances of surviving to reproduce again become minimal (Clutton-Brock, 1984). At the same time, older individuals might reduce reproductive investment due to senescence, or physiological loss of function with age (Monaghan *et al.*, 2008). If individuals are characterized by their physiological condition, rather than age per se, theoretical models have shown that apparent age-related declines can reflect adaptive reproductive restraint rather than constraints of senescence (McNamara *et al.*, 2009). Reduced reproductive investment can be adaptive if this slows down subsequent damage accumulation and allows individuals to reproduce successfully before death occurs from largely intrinsic causes. Experimental work on burying beetles *Nicrophorus vespilloides* has shown that both perceived risk of death and physiological costs of reproduction can explain complex patterns of age-related changes in investment (Cotter *et al.*, 2011). Beyond this study, there have been few empirical tests to bring together predictions from these different theories.

Viviparous tsetse flies (*Glossina* spp.) provide an interesting model to investigate age- and state-dependent reproductive investment (Langley & Clutton-Brock, 1998). Tsetse fly mothers show high levels of reproductive investment in each individual offspring, as they reproduce through adenotrophic viviparity (Benoit *et al.*, 2015). A single egg hatches as a larva in its mother's uterus and develops through three larval instars, during which it receives

all of its nutrition through milk-like secretions from a modified accessory gland, until it is ready to pupate as a late third instar larva. Mothers can produce a dozen or more offspring, singly, over their lifespan. The genus *Glossina* has thus evolved a reproductive strategy whereby live larvae are produced that do not feed independently: instead they use the fat and protein provided by the mother to complete the whole of puparial development and to produce a teneral adult with the full linear dimensions of a mature fly. With this strategy, the implicit demand for fat and protein from the mother is so large (the deposited larva typically weighs as much as the female that has just deposited it, Hargrove & Muzari, 2015b) that there is no possibility for a female to produce more than one offspring at a time.

Organisms such as tsetse flies, which always produce their offspring singly (monotocous) and produce multiple offspring in their lifetime (iteroparous), also present a special opportunity to understand the widespread correlation between maternal size and offspring size. This is because per-brood reproductive effort is equivalent to offspring size. Across a great diversity of species, there is a common trend that – within species or populations – larger mothers tend to produce larger offspring (Fox & Czesak, 2000; Roff, 2002; Marshall *et al.*, 2010; Rollinson & Rowe, 2015). A recent meta-analysis across 231 species of animals, for example, found that the correlation coefficient in intraspecific studies relating maternal size to offspring size is consistently positive across taxa, life histories and environments (Lim *et al.*, 2014). In spite of this strong empirical evidence, there is no general theoretical consensus on why large mothers should produce large offspring (Rollinson & Rowe, 2015). Indeed, classic models predict that maternal size should not necessarily correlate with offspring size as a single optimal size is favoured (Smith & Fretwell, 1974). More recent extensions of these models have demonstrated certain conditions under which such a correlation within species exists: for example, when larger mothers are more efficient at transferring resources (Sakai & Harada, 2001), or when smaller mothers experience higher

survival or energetic costs of reproduction (Kindsvater & Otto, 2014; Filin, 2015). The widespread relationship between maternal size and offspring size remains a puzzle, however, and more studies are needed which take into account other aspects of maternal state, such as nutrition (Rollinson & Rowe, 2015).

Here, we use data from a unique study on wild tsetse flies to explore the relationship between maternal state (size, nutritional status and estimated age) and reproductive investment as measured by offspring size. A study on laboratory tsetse flies found that maternal investment did not change with age (Langley & Clutton-Brock, 1998), suggesting that females do not exhibit reproductive restraint, terminal investment or reproductive senescence. It is unclear, however, whether this result is determined by the high-nutrition conditions typical of laboratory flies, or how the effects of maternal age and state (i.e., size or nutrition) interact in influencing reproductive investment. Until recently, field investigations of maternal investment in tsetse flies have been limited by the difficulty of capturing females that are about to deposit a larva. Our aim in this study is to quantify state-dependent maternal investment using field data from a sampling method where mothers are caught during larviposition, thus allowing for simultaneous measures of the size, nutritional state and age of mothers and the size of the offspring they have just produced (Muzari & Hargrove, 2007; Hargrove & Muzari, 2015a, 2015b).

Measuring maternal investment under field conditions also requires a consideration of extrinsic environmental factors. Temperature is an important determinant of body size in ectotherms, generally (Forster *et al.*, 2012; Klok & Harrison, 2013), and is also likely to play a key role in influencing reproductive investment in tsetse flies, for two reasons. First, flies have reduced time available for feeding during hotter periods: when temperatures rise above 32^0C, adults enter dark sheltered places and show reduced flight activity until temperatures decline in the late afternoon (Torr & Hargrove, 1999). Second, as they are poikilotherms,

their metabolic rate rises with temperature (Hargrove & Coates, 1990) and they digest their blood meal more quickly. Both of these factors might be expected to result in the production of smaller offspring as temperatures increase. As a consequence, when investigating the relationship between maternal state and reproductive investment in wild tsetse flies under natural conditions, we take into account these predicted effects of temperature. As tsetse flies are vectors of trypanosomes which cause nagana in livestock and sleeping sickness in humans, understanding the factors determining offspring size – a trait strongly linked to survival, particularly in warmer months (Phelps & Clarke, 1974) – might lead to improved predictions of the population dynamics of this important disease vector.

Materials and methods

Study site and sampling procedure

We used records from ovarian dissections of female *Glossina morsitans morsitans* Westwood collected near Rekomitjie Research Station, Zambezi Valley, Zimbabwe (16° 18' S, 29° 23' E) between September and November, in 1998 and in 1999. These are the hot dry months leading up to the summer rains. Data were obtained from females trapped in devices designed to mimic warthog burrows. The devices, described by Muzari and Hargrove (2007) and Hargrove and Muzari (2015a; b), were constructed out of 200-litre steel drums, which were cut, welded and buried in the ground to produce burrows with an entrance flush with ground level. Tsetse entering the burrow were caught in a trap consisting of an 8-gauge wire frame trap, covered with fine black gauze, inserted into the mouth of the burrow (see Hargrove and Muzari 2015a for further explanation). Full-term pregnant flies deposited a larva in the cage or, more commonly, were removed from the trap prior to larviposition and either deposited a larva prior to dissection or retained the late third-instar larva *in utero* until they were dissected.

Summary of offspring and maternal state variables

We used offspring dry weight as our measure of offspring size, which – in tsetse flies – is equivalent to total reproductive investment in a given breeding attempt. To investigate how maternal size, physiological condition, nutritional state, and estimated age influence reproductive investment, we measured the following variables in adult females: (i) thoracic residual dry weight (TRDW, i.e., the weight of the thorax after all fat has been extracted), which – although it increases rapidly in teneral flies after eclosion – is relatively invariant from the time a female is ready to produce her first larva (Hargrove & Muzari, 2015a), thus providing a reliable measure of adult size; (ii) fat content, which gives a measure of maternal physiological condition; (iii) haematin level, which declines approximately exponentially following feeding (Randolph *et al.*, 1991), and thus can be used to estimate the time since a fly last fed and (iv) age as estimated by ovarian dissection and the degree of wing fray (see below for details).

Processing of adult females and pupae

The head and legs of adult females were removed and discarded prior to processing. The wings were then excised and fixed, with transparent adhesive tape, to the dissecting record form so that wing length and fray (Jackson, 1946) could be measured. The female's ovaries and uterus were then dissected in saline solution under a binocular microscope as detailed by Hargrove (2012). Tsetse flies have paired ovaries with two ovarioles in each ovary. A single oocyte develops in each ovariole and, since ovulation occurs in a strict sequence and at regular intervals of about nine days, the relative sizes and positions of the oocytes indicate the number of times the fly has ovulated and thus the fly's age (Saunders, 1960; Challier, 1965). This system only works, however, for flies that have ovulated fewer than $i = 4$ times. For $i > 3$

ovulations, it is not possible to distinguish between a fly that has ovulated *i* times or *i*+4*n* times (as explained in Hargrove, 2012). We therefore used wing fray as additional information to estimate fly age, as older flies have more damaged wings, on average. Wing fray was categorized from 1 (perfect condition) to 6 (large rounded indentations to the wing) (Jackson, 1946). Following Hargrove (2012), we assumed that, for flies in ovarian category 4+4*n* to 7+4*n*, $n = 0$ if wing fray was less than or equal to 4, and $n = 1$ if wing fray was 5 or 6. We considered ovarian category (1–11) in our main analysis but, given that there is some error associated with this age estimation, we repeated our analysis using two more conservative methods of age categorisation. First, we used only categories from the first ovarian cycle, grouping those that had ovulated four or more times into a single category (i.e., ovarian category 1, 2, 3 and 4+), and, second, we considered three broader categories according to ovarian cycle (categories 1–3, 4–7 and 8–11; Appendix B).

Once ovarian dissection was complete, the reproductive organs (other than third instar larvae) were reinserted back into the abdomen. Maternal dry weight was assessed by drying the thorax and abdomen initially over calcium chloride and then in an oven at 70^{o}C over silica gel and weighing the fly on a Mettler AT-201 electronic balance (Mettler-Toledo, Greifensee, Switzerland) with a precision of 0.01 mg. Fat was then extracted using chloroform over 72 hours and flies were re-dried and reweighed to measure the residual dry weight (dry weight without fat). The thorax was removed for specific measurement of the TRDW, which is highly correlated with wing length (Hargrove & Muzari, 2015a) yet subject to less measurement error. TRDW provides a more reliable measure of adult size than the residual dry weight of the entire fly, which varies with hunger stage (Bursell, 1960). Pupae and third instar larvae were subjected to the same analytical procedure to measure total offspring dry weight. Maternal haematin levels were assessed by processing the supernatant from the ground-up abdomen as explained in detail by Hargrove and Muzari (2015b).

We included data on mothers captured in the burrows, and any offspring which could be attributed unequivocally to them – and which were either deposited in the burrow (n=13) or in the tube to which the full-term mother was transferred on capture (n=144). We also included data on 174 third-instar larvae dissected from mothers whose estimated percentage of pregnancy completed was >95%, indicating that these larvae were about to be deposited. There was no significant difference between dry weights of pupae collected in burrows and those deposited in tubes (mean ± SD pupal weight in mg: burrow, 8.90±0.38; tube, 8.98±0.08; Wilcoxon rank sum test, W = 977.5, P = 0.794). Moreover, there was no difference in dry weight between pupae and third-instar larvae dissected from mothers (mean ± SD weight in mg: pupae, 8.98±0.08; larvae 8.89±0.08; W = 14275.0, P = 0.479). We therefore combined data on larvae and pupae in our analyses.

Daily minimum and maximum temperatures were recorded at the field site using a Stevenson screen. Although the artificial burrows sampling method is only effective during the hot-dry months in the year, the mean daily temperature (i.e., the average between minimum and maximum recorded temperatures) varied from 24.7^0C to 34.5^0C over the sampling period. Accordingly, we accounted for potential temperature effects on offspring size by measuring the 9-day running mean temperature for the period prior to the day on which each mother-offspring pair was sampled. We selected this period based on the average duration of the pregnancy cycle: thus the 9-day running mean temperature is representative of conditions experienced during pregnancy (Hargrove, 1994).

Statistical analysis

We assessed the relationship between maternal factors and offspring size using multiple linear regression. All analyses were performed using R (R Development Core Team, 2012). We present parameter estimates, standard errors, *t* statistics and *P* values for all terms included in

the full model, as recommended by Forstmeier and Schielzeth (2011). To confirm our results, we also conducted stepwise model selection, by comparing the fit of models with different variables and sequentially removing those terms which did not significantly improve the fit of the model to the data (see Crawley, 2007; Appendix C). We considered offspring dry weight (puparial or third-instar larval) as our response variable, and the following input variables in the full model: maternal TRDW, fat, age category and haematin content, 9-day running mean temperature and year of capture. Continuous variables were mean-centred and standardized to facilitate comparison of parameter estimates – which were measured on different scales – within the same linear regression model (Schielzeth, 2010). We first examined correlations among the input variables, asking specifically whether maternal condition traits changed with maternal age or depend on environmental factors (Appendix A). We calculated the variance inflation factors of the combination of variables to ensure that any correlations among variables did not create collinearity in our multivariate model (Zuur *et al.*, 2009).

Results

We found strong positive correlations among maternal size and condition: flies with higher TRDW also had higher fat content (Figure 1, Table A1 in Appendix A). Older mothers had higher TRDW than younger ones, presumably because these individuals themselves were born during cooler months of the year (June and July) when flies tend to emerge at a larger size (Hargrove, 1999). There was a marginal, but not significant, increase in fat and haematin with age (Figure 2, Table A1 in Appendix A). Maternal TRDW, fat and haematin levels tended to be higher in 1999 than in 1998 (Figure A1 and Table A1 in Appendix A), yet there was no effect of 9-day running mean temperature on these size or condition measures. In spite of these correlations, the variance inflation factor among input variables was less than 1.5 so all terms were included in the multivariate analysis.

Mothers with higher fat and thoracic residual dry weight produced heavier offspring (Table 1, Figure 3b and c), and those with higher haematin levels produced lighter offspring (Table 1, Figure 3d). When we considered all 11 ovarian age categories, there was no directional effect, once we had adjusted for fly size, of estimated age on offspring weight, although offspring in categories 6 produced significantly heavier offspring than those in category 1 (Table 1, Figure 4a). When we considered four levels of ovarian age category (1, 2, 3, 4+) or just three (1–3, 4–7 and 8–11), we did not find any significant effect of these more conservative estimates of maternal age on offspring size (tables B1 and B2). In both cases, however, there was a marginal effect ($0.05 < P < 0.1$) that older females produced larger offspring. Mothers produced smaller offspring when the mean temperature during the nine-month pregnancy period was higher (Table 1, Figure 4b). We did not find any significant difference in offspring dry weight across the years (Table 1).

Discussion

In this study, we found that wild tsetse mothers that were larger and had more fat in turn produced larger offspring and thus invested more in their young. Females who had recently ingested a blood meal produced smaller offspring and, as has been shown in earlier work, smaller offspring were produced under hotter conditions. We found no strong evidence that investment changed with age, although there was a weak increase in the size of offspring produced by older females. These findings reveal insights about the life history of a disease vector, whose reproductive ecology in natural conditions has, until recently, been relatively poorly understood.

We found that larger mothers with higher fat contents invest more in their offspring in terms of producing heavier young. Such a result could arise through a variety of mechanisms: intrinsically larger females may produce bigger offspring through genetic effects, or because

265 they cope better with the energetic costs of pregnancy and can allocate more reserves to their
266 developing young (Reznick *et al.*, 2000). Alternatively, females may be making strategic
267 decisions based on their nutritional state about how much to allocate to their young, and these
268 decisions may vary from one pregnancy to another within their lifetime (reviewed in
269 Rollinson & Rowe, 2015). Our finding that blood-meal content negatively correlates with
270 offspring size supports this latter body of theory: females who find a meal quite late in
271 gestation simply do not have enough time to transfer fat and protein to their current larva
272 (Hargrove, 1999) and may thus save these reserves for future reproduction. It is also possible
273 that females who feed late in pregnancy are themselves in poor intrinsic condition, although
274 we did not find any negative correlation between haematin content and fat. Our findings
275 provide partial support for the suggestion by Rollinson & Rowe (2015) that the overwhelming
276 empirical evidence linking maternal phenotype with offspring size (e.g., Lim et al., 2014) is
277 more likely driven by underlying condition (e.g., nutritional status, general health) than by
278 maternal size alone. Further study is needed to determine the ultimate reason why the
279 condition-offspring size relationship exists.

280 Once we controlled for variation in maternal nutritional state and size, we did not find
281 any strong effect of estimated maternal age on offspring size, although older females tended
282 to produce larger offspring. Almost two decades after a laboratory study demonstrating a lack
283 of age-related changes in tsetse reproductive investment (Langley & Clutton-Brock, 1998),
284 our results present a test of the explanation that such results might be an artefact of the high
285 nutritional conditions in the laboratory. With the important caveat that ovarian age estimation
286 is an uncertain measure, particularly for females who have ovulated more than four times, our
287 results indicate that reproductive senescence or restraint may be absent in wild tsetse flies too,
288 which contrasts with several studies on other invertebrates in which older females produce
289 smaller eggs (Karlsson & Wiklund, 1984; Boggs, 1986; Giron & Casas, 2003). Note that our

results were limited to the hotter months of the year, which are the only months when females use underground burrows to deposit their larvae. The slight increase in investment with age may therefore reflect the fact that the older individuals in our dataset were born in cooler months of the year. This means they tend to be larger than average (Hargrove, 1999), and – even though we adjusted for size in our analysis – they may also exhibit higher unmeasured levels of physiological condition not fully explained by variation in TRDW. Whether such effects are generalizable to other months of the year are currently unknown. Moreover, in light of a recent study on the larger sympatric species *Glossina pallidipes* Austen caught using the same artificial burrows methods, mothers delay investing protein and fat in their offspring until much later in gestation than in other viviparous organisms (Hargrove & Muzari, 2015a). As such, it is possible that there are age-related changes in the rates of terminated pregnancies, which would be a less costly form of adjusting investment than producing smaller offspring at birth. This possibility should be tested in future studies.

Our results highlight the importance of considering both intrinsic and extrinsic state variables when measuring maternal investment. We find that mothers produce smaller offspring when they experienced hotter conditions during pregnancy. Previous field studies have shown that smaller pupae tend to be produced during the warmer seasons in the year, and smaller individuals are particularly prone to mortality at these times (Jackson, 1948; Glasgow, 1961; Phelps & Clarke, 1974). Whether being born small under hot conditions is adaptive (Forster *et al.*, 2012), akin to the thrifty phenotype suggested for small-for-gestational-age humans born under harsh conditions (Wells, 2007), or a constraint due to heat stress on mothers, requires further experimentation in the laboratory. Our results are restricted to the hot period of the year, which is the only time of the year when tsetse use burrows for larviposition. Whether they are robust to less stressful thermal conditions would be difficult to

test in the wild, until new trapping methods are developed to sample, simultaneously and across a range of conditions, tsetse pupae and the mothers that produced them.

In conclusion, we present here a unique study on wild, viviparous tsetse flies in which the link between maternal physiological state and offspring size is quantified under natural conditions. Flies adjust their investment according to their physiological state and the abiotic environment: females who are larger, in better condition and experiencing cooler temperatures produce larger offspring. We use a relatively sensitive method to estimate age and do not find any strong overall trend of age-related investment, although older females tend to produce slightly larger offspring. Such results are in line with an earlier laboratory study, which was not able to preclude the possibility that high nutrition obscured any age-related decline in investment that might have happened in the wild. However, due to constraints on our study we explain what further work is required to explore this topic in the future. We suggest that tsetse flies might strategically adjust offspring size – perhaps because there is strong selection against being born too small – but also that variation in size could also occur due to constraints on female reproductive investment. Future experimental work and detailed sampling under a range of conditions are required to tease apart the relative influences of strategic adaptation and constraint on maternal investment decisions.

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Contribution of authors SE and JWH designed the study, based on existing data collected by JWH and the tsetse capture and dissection team at Rekomitjie Research Station; SE, HC, EG and JWH analysed the data; SE wrote the paper with input from JWH.

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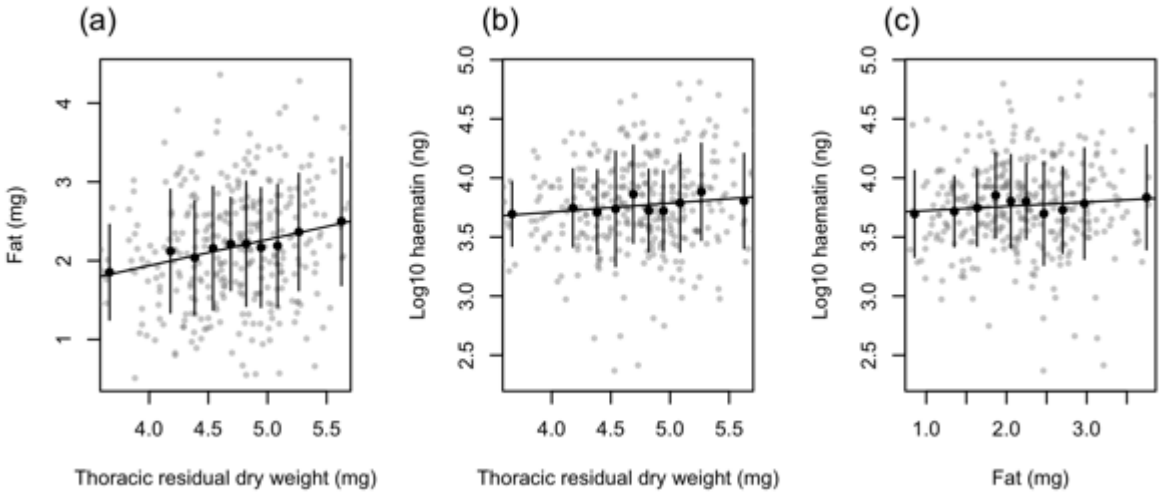
446

447 **Table 1.** Parameter effect estimates and standard error (with associated *t*-statistic and *P*-
448 value) for terms included in a multivariate general linear model (GLM) analysing variation in
449 offspring dry weight. Terms in bold are those considered statistically significant at the $P<0.05$
450 threshold. All continuous input variables were standardized, and levels for categorical
451 variables are given relative to the reference level (Ovarian category 1, Year 1998).
452

Explanatory term	**Estimate**	**Std. Error**	**t-value**	**P-value**
(Intercept)	875.5	15.3	57.3	<0.0001
Thoracic residual dry weight	**17.2**	**5.29**	**3.25**	**0.001**
Fat	**36.1**	**5.16**	**7.00**	**0.000**
Log(10) haematin	**-10.2**	**5.13**	**-2.00**	**0.047**
Category 2	6.6	19.8	0.33	0.741
Category 3	23.8	21.1	1.13	0.260
Category 4	20.1	18.6	1.08	0.280
Category 5	23.8	20.7	1.15	0.251
Category 6	**68.3**	**24.3**	**2.81**	**0.005**
Category 7	7.4	28.8	0.26	0.797
Category 8	43.0	23.7	1.81	0.071
Category 9	24.4	23.5	1.04	0.301
Category 10	29.9	25.7	1.17	0.245
Category 11	29.9	32.1	0.93	0.352
Temperature	**-17.0**	**5.0**	**-3.38**	**0.001**
Capture year (1999)	-15.1	11.48	-1.32	0.188

453

454 **Figure 1.** Correlations among maternal state variables. Shown in black are the mean ± SD
455 levels (binned categories) and the regression line from a simple linear model relating the two
456 terms in each plot. The raw data points are plotted in light grey. Only the correlation between
457 TRDW and fat was significant (see table A1 for correlation coefficients). Bin widths were
458 chosen to reflect equal distribution of the data, with about 30 data-points in each bin.



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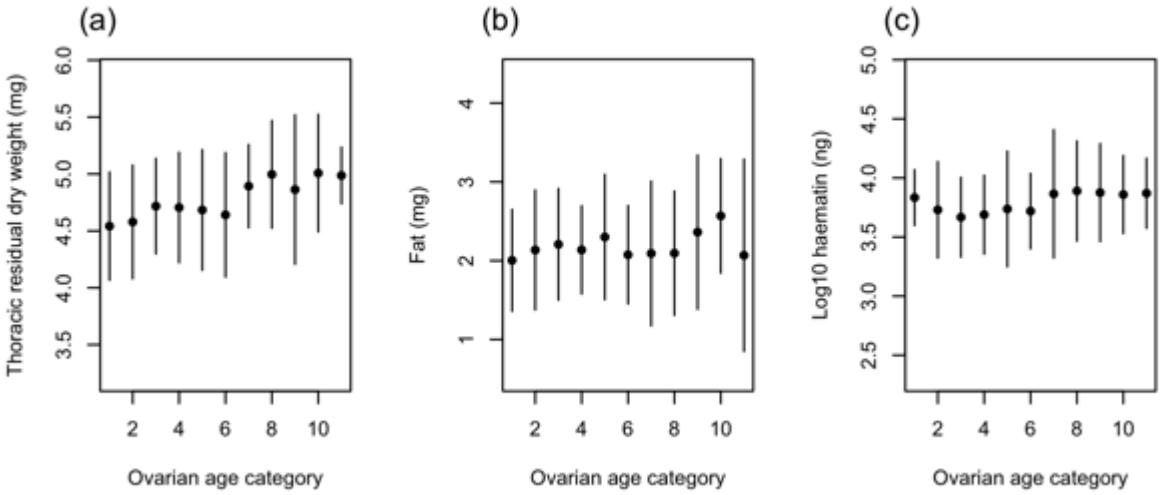
460

461 **Figure 2.** Relationship between maternal age (estimated through ovarian dissection, see text)
462 and maternal state, as measured by (a) thoracic residual dry weight, (b) fat content and (c)
463 haematin. Points show the mean ± SD levels for each ovarian category. There was a
464 statistically significant effect of ovarian age category on TRDW but not on fat or haematin
465 content (see results in table A1).



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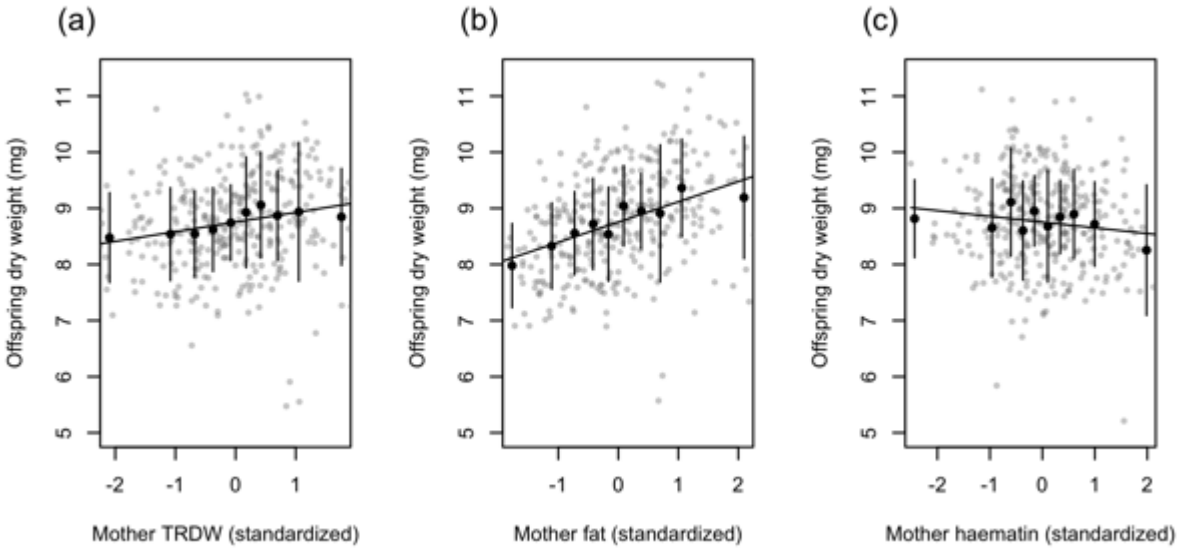
467

468 **Figure 3.** Relationship between offspring dry weight and maternal state, as measured by (a)
469 thoracic residual dry weight, (b) fat content and (c) haematin. Shown in black are the
470 predicted effects from the full model (line) and mean ± SD partial residuals controlling for
471 other factors in the model (bin widths were chosen to reflect equal distribution of the data,
472 with about 30 data-points in each bin). Raw partial residuals are plotted in light grey.



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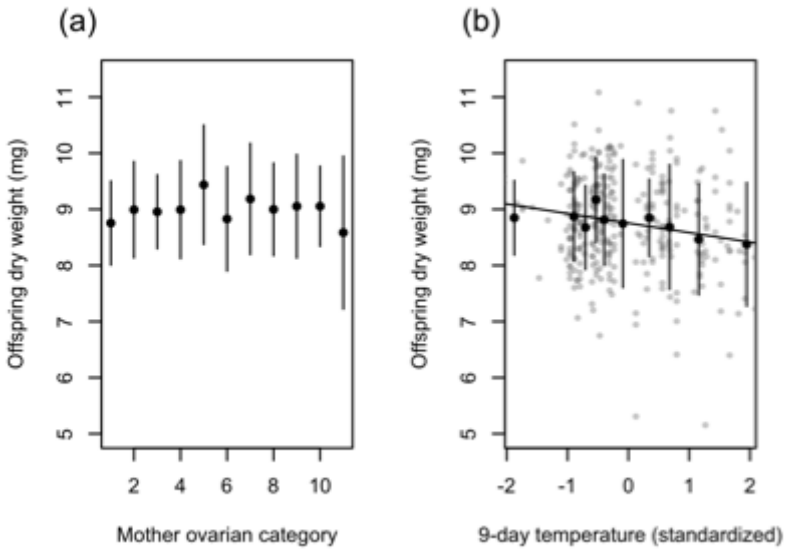
475 **Figure 4.** Relationship between offspring dry weight and (a) maternal age, estimated by
476 ovarian category, and (b) 9-day mean temperature during pregnancy period. Shown in black
477 are the predicted effects from the full model (line, b only) and mean ± SD partial residuals
478 controlling for other factors in the model (bin widths in [b] were chosen to reflect equal
479 distribution of the data, with about 30 data-points in each bin). Raw partial residuals are
480 plotted in light grey.



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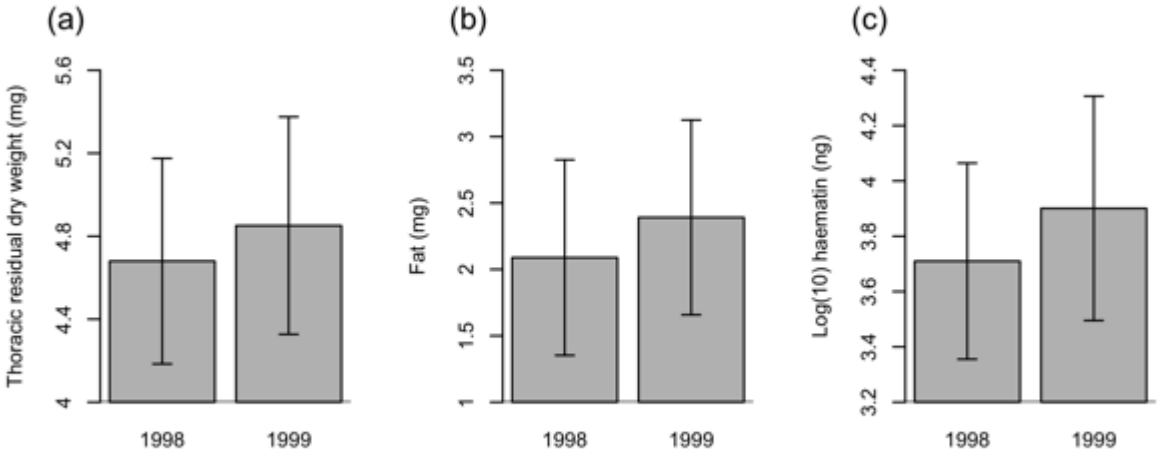
Appendix A: Correlations among explanatory variables

We examined correlations among all pairs of maternal state variables (thoracic residual dry weight, fat, log(10) haematin) and between each variable and estimated age, temperature and year. For pairs of continuous variables, correlations were measured by calculating the Pearson's product moment correlation coefficient using the function cor.test in R. For examining the effect of categorical variables (ovarian category and year), we conducted an ANOVA on the trait of interest with only the categorical variable of interest as a covariate. Results of both types of tests are provided in table A1 below.

Table A1. Correlations among maternal state variables and effect of age and environmental factors. Shown are the correlation coefficients (*r*) between pairs of continuous variables, or results from *F*-tests from an ANOVA including a single categorical variable. For both tests, the statistical significance of the relationship is depicted by the number of asterisks (***: P<0.001; **: 0.001<=P<0.01; *:0.01<=P<0.05). All tests were based on n = 331 observations.

	TRDW	**Fat**	**Log(haem.)**
TRDW	---		
Fat	r = 0.23***	---	
Log(haem.)	r = 0.10	r = 0.07	---
Temperature	r = -0.06	r = 0.03	r = -0.01
Ovarian category	F = 2.95**	F = 1.12	F = 1.47
Year	F = 7.98**	F = 11.7***	F = 18.3***

499 **Figure A1.** Variation in (a) thoracic residual dry weight, (b) fat content and (c) haematin
500 across years. Bar plots show mean ± SD. Note that the sample size in 1998 (n = 233) was
501 higher than in 1999 (n = 98).



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Appendix B: Results of analysis using conservative ovarian categorization

In the main text, we used a combination of ovarian category and wing fray to assign individuals to age categories (from 1 to 11). Here, we repeat our analysis using a more conservative approach in which we do not further partition those flies which have ovulated at least four times (i.e., categories 1,2,3,4+). We also repeat the analysis using the combined ovarian category and wing fray approach, but grouping females into three categories (ovarian category 1–3, 4–7 and 8–11), thus approximating 'young', 'middle-aged' and 'old flies', to examine broader patterns. As with the analysis of the main text, these additional analyses do not show any significant effect of a more conservative estimation of maternal age on offspring size (tables B1 and B2), although in both cases there is a marginal effect ($P < 0.1$) that older females produce larger offspring. These analyses confirm that the lack of a significant effect of maternal age in our main analysis is unlikely to be due to the uncertainty in estimating the age of flies which have ovulated more than 3 times, or by fitting too many levels of a categorical variable to a relatively small dataset.

Table B1 and B2. Effect size and standard error (with associated *t*-statistic and *P*-value) for terms included in a linear model analysing variation in offspring dry weight, with age categorised as 1,2,3,4+ (Table B1) or 1–3, 4–7 and 8–11 (Table B2). Terms in bold are those considered statistically significant at the P<0.05 threshold. All continuous input variables were standardized, and levels for categorical variables are given relative to the reference level (Ovarian category 1, 1998 and September).

525 **Table B1**

Explanatory term	Estimate	Std. Error	t-value	P-value
(Intercept)	886	9.23	96.0	<0.0001
Thoracic residual dry weight	**17.4**	**5.24**	**3.32**	**0.001**
Fat	**36.0**	**5.08**	**7.09**	**<0.0001**
Log(10) haematin	**-10.9**	**5.08**	**-2.14**	**0.033**
Category 4–7	17.4	11.3	1.55	0.123
Category 8–11	22.6	13.6	1.66	0.098
Temperature	**-16.9**	**5.0**	**-3.42**	**0.001**
Capture year	-16.2	11.3	-1.43	0.154

526

527 **Table B2**

Explanatory term	Estimate	Std. Error	t-value	P-value
(Intercept)	**875**	**15.3**	**57.4**	**<0.0001**
Thoracic residual dry weight	**17.2**	**5.17**	**3.33**	**0.001**
Fat	**35.6**	**5.09**	**7.01**	**<0.0001**
Log(10) haematin	**-10.1**	**5.06**	**-1.99**	**0.048**
Category 2	6.6	19.8	0.34	0.738
Category 3	24.3	21.1	1.15	0.249
Category 4+	29.2	16.1	1.82	0.070
Temperature	**-16.2**	**5.0**	**-3.24**	**0.001**
Capture year	-15.3	11.4	-1.34	0.181

528

529

Appendix C: Results of multivariate analysis using model simplification

In the main text, we present results in which we include all terms of interest in our multivariate model (even those deemed to not have a significant effect). We appreciate that there are other approaches to interpreting the significance of effects from multivariate analysis. Below, we present results from one such alternative in which only significant terms are retained in the model (based on stepwise model selection in which terms are removed from the model based on F-statistic of each effect). We then examine whether the inclusion or exclusion of particular terms significantly improves the fit of this minimal model to the dataset. Results from this analysis are provided in Table C1, and it is clear that the same terms remain significant whether non-significant terms are retained (main text), or removed from the multivariate model.

Table C1. Results of model selection either dropping terms included in the minimal model or adding those non-significant terms which were not retained in the minimal model.

Term	In minimal model?	F-value	Df	P-value
Fat	**Yes**	**48.5**	**1**	**<0.0001**
Log(haematin)	**Yes**	**5.82**	**1**	**0.016**
TRDW	**Yes**	**13.4**	**1**	**0.0003**
Temperature	**Yes**	**11.8**	**2**	**0.0007**
Ovarian category	No	1.2	10	0.292
Capture year	No	2.7	1	0.104